Oxidative DNA Damage – the possible use of biomarkers as additional prognostic factors in oncology

Krzysztof Roszkowski

1Department of Radiotherapy, the F. Lukaszczyk Oncology Center Bydgoszcz, Romanowskiej 2, 85-796 Bydgoszcz, Poland

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Oxidative modification of DNA bases
4. Oxidative DNA damage repair mechanisms
4.1. Diet as a potential source of 8-oxoGua and 8-oxodG content in urine
4.2. Cell death as a potential source of 8-oxoGua and 8-oxodG content in urine
5. The effect of antioxidant supplementation on the levels of oxidative DNA lesions
6. Potential application of oxidative DNA damage determination in clinical practice
   6.1. Chemotherapy
   6.2. Radiation therapy
   6.3. Oxidative DNA damage repair products as molecular markers of cancer
7. References

1. ABSTRACT

Oxidative stress represents a deregulation of the homeostasis between the reactive oxygen species and the mechanisms of detoxification and repair. By analyzing the level of oxidatively damaged DNA bases and nucleosides in urine we can assess the extent of DNA repair within the whole body. High levels of markers of oxidative DNA damage excreted in the urine indicate elevated levels of oxidative stress, but can also reflect a high level of efficiency of the processes that work to repair this damage (oxidative stress can be high and the repair processes eliminate its effects). In the present review we discuss the role of oxidative stress, oxidative DNA damage repair mechanisms, potential sources of 8-oxoGua and 8-oxodG (basic markers of oxidative stress) content in urine, effect of antioxidant supplementation on the levels of oxidative DNA lesions, potential application of oxidative DNA damage determination in clinical practice.

2. INTRODUCTION

Oxidative stress represents a deregulation of the homeostasis between the reactive oxygen species and the mechanisms of detoxification and repair. Reactive oxygen species (ROS) are produced during the metabolic cycle of every aerobic cell and consist of an atom of oxygen and an unpaired electron. ROS react with the most important structures of cells and particles altering their biological function (1,2). Similarly, reactive nitrogen species (RNS) create toxic products through their interaction with cells and particles. Both ROS and RNS play important roles not only in the process of energy production, lipid peroxidation, oxygenation, nitration, and nitrozylation of proteins and of DNA, but also in the body’s response to catecholamines. The particles are inactivated by natural antioxidants (e.g. beetroot). Excessive production of ROS and RNS results in both oxidative and nitric stress both of which are involved in various pathological conditions.
typical of neoplasms, neuro-degenerative disorders, viral, toxic, or inflammatory processes (1). Deregulation of the homeostasis between reactive oxygen species and the mechanisms of detoxification in cells leads to the destruction of tissue and the development of pathology, that is neoplasm, and after wards participates in tumor progression (2). These radicals may also influence the signal transduction cascade in the cell, activating such transcription factors as NFκB and AP-1 (plasminogen activator) that control the immediate cell response to stress (3). Pro-neoplastic activity of these radicals results not only from DNA damage (4), but also from the damage of proteins and lipids, as the modification of these particles may increase the risk of mutation (5). The factors neutralizing free radicals prevent DNA damage and mutagenesis (1). Because radiotherapy results in the ionization of DNA and of water, it stimulates the development of hydroxyl radicals such as hydrogen peroxide, hydrated electrons, and other oxygen-free radicals. All of these are highly reactive and capable of interacting with DNA and causing damage. It has thus been proposed that the levels of oxidative DNA damage markers in urine might, on one hand, be predictive of the intensity of the oxidative stress level in patients undergoing radiotherapy, but on the other hand, they may reflect the efficiency of repair mechanisms.

3. OXIDATIVE MODIFICATION OF DNA BASES

Every aerobic cell produces a certain quantity of reactive oxygen species (ROS) during its metabolic cycle (6). ROS are responsible for various types of damage to both nuclear and mitochondrial DNA. Their main effects include the oxidation of DNA bases, depurination, and DNA strand breaks (7).

Under normal physiological conditions, the main species responsible for genomic damage is the hydroxyl radical .DNA most commonly lead to damage to DNA bases, thus generating a number of DNA base derivatives. Major ROS-induced derivatives of DNA bases are the following: guanine is transformed into 8-oxoguanine (9) and FAPy-guanine (2,6-diamino-4-hydroxy-5-formamidopyrimidine); adenine is transformed into 8-hydroxyadenine, 2-hydroxyadenine, and FAPy-adenine (4,6-diamino-5-formamidopyrimidine); cytosine is transformed into 5-hydroxycytosine, 5-hydroxyuracil and 5,6-dihydroxyuracil; and thymine is transformed into thymine glycol, 5-hydroxymethyluracil and 5-hydroxymethylhydantoine (10,11).

Till now, more than 20 different types of oxidative modifications of DNA bases have been identified (12). Data from the literature demonstrates that tumor tissues are characterized by elevated levels of H₂O₂. The ease of diffusion through biological membranes allows H₂O₂ to enter the nucleus and generate hydroxyl radicals . The levels of free radicals within the body vary over time; therefore, aerobic organisms evolved to develop a number of mechanisms to adapt to such variable conditions, synthesizing anti-oxidative enzymes and/or enzymes that repair oxidative DNA damage (15,16).

It is established and widely accepted fact that a certain degree of oxidative modification of DNA is also present in normal cells (17). This is a manifestation of the balance between the formation of ROS which attack DNA in the course of numerous metabolic processes and the elimination of these lesions by specific DNA repair enzymes. Presently we do not know how high the endogenous level of these potentially mutagenic lesions can be. The authors, using different analytical techniques, now report values ranging from 0.2 – to several modifications/10⁶ of base pairs for normal cells (18,19). It seems, however, that this level varies significantly among individuals (20,21). In the cell, there is a balance between the production of ROS, which is responsible for the oxidative damage of DNA, and the elimination of the resulting lesions. The activity of ROS results in the accumulation of oxidative DNA lesions within the entire body; these are repaired through restorative processes and removed into urine. A certain number of lesions, however, stay in the body; these constitute the so-called background level, characteristic for each organism and dependent on the effectiveness of repair (22). By analysing the level of DNA oxidative damage in the urine we can assess the extent of repair within the whole body. High levels of markers of oxidative DNA damage excreted in urine indicate elevated levels of oxidative stress, but can also reflect a high level of efficiency of the processes that work to repair this damage (oxidative stress can be high and the repair processes eliminate its effects). Clearly, combining data about the basic level typical for each person with the results of the analysis of 8-oxoGua and 8-oxodG levels excreted in urine could provide much-needed information regarding the mechanisms of DNA repair.

4. OXIDATIVE DNA DAMAGE REPAIR MECHANISMS

As many experimental studies have definitively demonstrated, the level of 8-oxodG in urine is a particularly sensitive marker of oxidative stress. Moreover, it has been assumed to reflect enzyme activity involved in the nucleotide excision repair (NER) processes or of the hMTH protein that hydrolyzes 8-oxodGTP (23,24).

The main oxidative DNA damage repair mechanism, however, is base excision repair (BER). This was confirmed by the discovery and successful cloning of specific glycosylases that identify and eliminate modified DNA bases from DNA (25). The role of NER mechanisms is only supportive in case of the oxidatively damaged DNA bases (26,27).

Human cells are equipped with three levels of protection. The first level is the human homologue of MutT (hMTH1). The enzyme hydrolyzes 8-oxodGTP into 8-oxodGMP, thus preventing the use of abnormal nucleoside triphosphate as a substrate for DNA polymerase. The second level of protection consists of specific glycosylases that initiate base excision repair (BER) processes. Finally,
human homologue of Mut Y (hMYH) eliminates adenine that was erroneously paired with 8-oxoGua (28,29) (Figure 1).

One might expect that the analysis of urinary levels of 8-oxoguanine (i.e., a modified base), as a product of glycosylase activity, would allow a more complete assessment of DNA repair processes. However, with few exceptions, studies concerning this issue have been devoted to 8-oxodG detection (22). This has mainly been due to methodological problems (sample analysis is extremely complicated). These problems were solved by Prof. R. Olinski and his team from Collegium Medicum of the Nicolaus Copernicus University in Bydgoszcz. The implemented technique enabled a simultaneous analysis of the modified base and nucleoside in one urine sample. The technique involves initial purification of the fractions containing modified compounds using high performance liquid chromatography (HPLC), followed by the analysis of these fractions using gas chromatography/mass spectrometry (GC/MS) technique. For a precise quantitative and qualitative assessment, analogues of the studied molecules labelled with stable isotopes were used (30,31).

4.1. Diet as a potential source of 8-oxoGua and 8-oxodG content in urine

Some studies have demonstrated that diet may affect the levels of 8-oxoGua in urine (32). In our study, the urine samples (daily urine collection) were collected over a period of 3-5 days from the same group of 24 subjects on a diet free of nucleic acids and again upon the return to a normal, unrestricted diet. The mean levels of 8-oxodG and 8-oxoGua in the urine samples collected from the studied groups of subjects were comparable regardless of the type of diet and, in the case of the experimental group, it was concluded that the urinary excretion of those molecules did not depend on diet (33). In another study, different amounts (up to 25 mg) of oxidatively modified DNA, $^{15}$N labelled, were taken orally by volunteers. Next, blood and urine samples were collected over a two-week period. No $^{15}$N labelled 8-oxoGua or 8-oxodG were found in the urine or DNA of peripheral blood monoclonal cells, collected from the same study participants (34), confirming that diet has no effect on the detectable levels of such lesions.

4.2. Cell death as a potential source of 8-oxoGua and 8-oxodG content in urine

It has been suggested that the urinary excretion of 8-oxodG represents DNA degradation originating from dead cells with delayed oxidation. The cooperation between ROS and cellular components can damage the biomolecules containing DNA. Studies of Faure et al. and Erhola et al. (35,36), did not find any increase in the urinary excretion of 8-oxodG, despite the obvious evidence of mass reduction in the treated cancers. Some reports indicate an apparent increase in the urinary excretion of 8-oxodG after radio-chemotherapy or radiotherapy alone (37,38). Measurements of the urinary excretion of only a repair product can sometimes be misleading as they provide no information about the body’s oxidative status (the rate of lesion formation vs. the rate of repair) in cellular DNA; they provide information only about the mean value reflecting the repair of past lesions.

5. THE EFFECT OF ANTIOXIDANT SUPPLEMENTATION ON THE LEVELS OF OXIDATIVE DNA LESIONS

Despite numerous studies on antioxidant supplementation during radiotherapy and much commentary, we have been unable to conclude whether the administration of antioxidants in patients during radiotherapy is safe. We therefore carried out this study in order to analyse for the first time a wide range of parameters reflecting oxidative DNA lesions (24-hour
urine excretion of 8-oxoGua (8-oxo-7,8-dihydroguanine) and 8-oxodG (8-oxo-7,8-dihydro-2-deoxyguanosine) as well as the levels of 8-oxodG in the DNA isolated from peripheral blood leukocytes. We analysed clinical results of patients undergoing radiation therapy and being supplemented with *Beta vulgaris*, which has strong antioxidant properties (39). In the subgroup of patients receiving supplementation, slightly lower acute reactions to radiotherapy were found during each week of radiotherapy using the Dische scale, as compared with the subgroup that did not receive supplementation. The median values of the overall survival rate for the supplemented patients were higher when compared with the values obtained from the control group -36.8 mo. and 26.1 mo. respectively. Supplementation with *Beta vulgaris* during radiotherapy, however, did not significantly affect the values of the assayed oxidative stress markers. During the first two weeks of radiotherapy the levels of 8-oxodG excreted in the urine were higher in those patients who received supplementation, but no differences were noted in the levels of the excreted 8-oxoGua and lower 8-oxodG values in cellular DNA over the entire period of treatment as compared with the values obtained from the control group. The results of the study showed that where a supplementation with *Beta vulgaris* in irradiated patients did not affect the long-term prognosis, it did decrease the intensity of post-radiation reactions without affecting the levels of oxidative stress markers during the treatment (39).

**6. POTENTIAL APPLICATION OF OXIDATIVE DNA DAMAGE DETERMINATION IN CLINICAL PRACTICE**

The collection of diagnostic materials from patients is restricted by certain issues, often of an ethical nature. The ideal method of collection would be non-invasive as well as technically feasible. Urine is a clinical material collected from patients in a non-invasive manner. This fact supports the use of analysis of modified bases in urine as the preferred diagnostic method in clinical practice.

The current practice of oncology, characterized by the widespread use of advanced treatment methods such as conformal radiation therapy and targeted chemotherapy, ideally could rely on markers to facilitate the "prognosis" of clinical outcomes at early stages of treatment or even prior to the initiation of treatment.

There is no doubt that both ionizing radiation and some chemotherapeutic agents used in cancer treatment produce ROS, both locally and systemically, leading to oxidative DNA damage. It is well known that markers of DNA damage, such as oxidatively modified bases and nucleotides 8-oxo-7,8-dihydroguanine (8-oxoGua) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) excised from DNA strands in the cellular repair processes, are excreted in the urine as unaltered compounds not subject to subsequent metabolism (22).

**6.1. Chemotherapy**

Cisplatin is one of the most effective chemotherapeutic agents used to treat many types of malignant tumors. It has generally been assumed that Cisplatin cytotoxicity is mainly caused by formation of inter- and intrastrand DNA crosslinks or monoadducts that inhibit DNA replication and/or transcription (40,41). The effect of oxidative stress is secondary, although important for the outcome of treatment.

Moreover, cisplatin-based chemotherapy is known to cause a decrease in antioxidant serum concentrations. This may result from the antioxidants being used up by the oxidative stress associated with chemotherapy as well as by the excess evaporation of water soluble small molecule antioxidants, such as uric acid (42). Once the balance between antioxidants and ROS is disturbed, the amount of oxidative damage can also change. Based on the analysis of several studies, it was found that the antioxidants present in dietary supplements in small doses had no effect on anti-neoplasms treatment (43). Oxidation products present in urine do not necessarily indicate damage to neoplastic cells, but they are indicative of the overall level of damage in the body (in the neoplastic cells and healthy tissues at the whole body level).

The available evidence suggests that the cytotoxic effects of cisplatin are directly associated with the induction of reactive oxygen species (ROS) (44,45). Siomek *et al.* (46) observed an increase in the urine levels of 8-oxodG and 8-oxoGua in most cancer patients 24 hours after infusion with cisplatin. At the same time, an increase in 8-oxodG levels in leukocyte DNA was observed, though of only marginal statistical significance. This increased elimination of 8-oxoGua is a manifestation of the successful repair of DNA damage. Moreover, 24 hours after the administration of cisplatin, the levels of 8-oxoGua increased by about 80%. It should be mentioned that damaged DNA bases are completely excreted from human cells within four hours (47). Several days after the administration of cisplatin, a significant reduction in 8-oxoGua levels as compared to the previous time, was observed. No return to the baseline value, however, was seen. In addition, as previously mentioned, 8-oxodG levels in the cellular DNA of some patients was significantly higher several days after treatment. Thus the variety of oxidative damage to DNA bases appears to be very broad.

The presence of repair products in cellular DNA may therefore prove significant, at least in some patients, as far as the efficacy of treatment and possible prevention of treatment complications (48).

Interestingly, most episodes of clinical remission were observed in those patients who responded with sharp increases of modified DNA base levels in urine 24 hours after the infusion of the drug with the values remaining at high levels in the samples collected several days after treatment had been initiated. No statistically significant changes in the urinary 8-oxoGua levels were found in the group that showed disease progression; however, the level of the other marker of oxidative DNA damage, 8-oxodG,
was significantly lower in the urine samples collected after the administration of the drug. This suggests not only reduced levels of ROS, but also the potential development of cellular resistance to the drug in this group of patients. A significant increase in the levels of 8-oxoGua excreted in urine after the first post-infusion day as compared to the pre-treatment values may suggest a positive clinical response to the treatment in cisplatin-treated patients. Lack of significant changes in the renal excretion of this derivative may indicate possible treatment failure.

The relationship between the effect of chemotherapy and oxidative stress has not yet been fully determined. Elevated levels of DNA oxidative damage products may be connected with neoplastic disease and its treatment as well as with other pathological conditions, such as inflammatory conditions. We should also keep in mind that inflammatory conditions predispose patients to neoplasm formation.

6.2. Radiation therapy

The interaction of photon and gamma radiation used in cancer therapy and water molecules (known as water radiolysis) generate free oxygen radicals/reactive oxygen species, particularly hydroxyl radicals. These are highly reactive; in particular they react with biomolecules to form most types of oxidative DNA damage (49). It is therefore possible that the oxidative DNA damage caused by radiation therapy is largely responsible for its therapeutic effects (Figure 2). As suggested by some literature reports and by our own data, the base excision pathway of hOGG1 glycosylase that eliminates 8-oxoGua from cellular DNA is responsible for the presence of 8-oxoGua in urine (50,51). An interesting report was published suggesting a significant reduction in 8-oxoGua repair activity within the DNA in head and neck cancer patients (52). As the authors of the study suggest, reduced activity of the main enzyme responsible for elimination of 8-oxoGua from the DNA should lead to accumulation of damage to cellular DNA. It is therefore possible that, at least in some patients with the lowest activity of OGG1, a combination of reduced activity of OGG1 and radiation therapy may be associated with increased levels of 8-oxoGua originating from cellular DNA. Apparently, the reduced activity of DNA repair is incapable of coping with the additional quantities of 8-oxo-DNA generated by the radiation, thus leading to an increase in instances of potentially mutagenic cellular DNA damage.

Reports published to-date suggests that the levels of oxidatively modified bases and nucleotides are independent of diet (33). The analysis of 8-oxoGua and 8-oxodG in urine samples collected from patients prior to radiotherapy at different times after irradiation should provide data, on one hand, about the effectiveness of the repair of oxidative DNA lesions and, on the other hand, about the
8-oxoGua- cancer biomarker and prognostic factor

Figure 3. Kaplan-Meier curves of 8-oxoGua in urine after the first fraction of radiotherapy (lung, breast, prostate, head & neck cancer) (54).

The intensity of oxidative stress at the level of the body. A comparison of the values reflecting the level of these modifications with the parameters assessing the success of treatment, such as the overall survival of the patients, should allow us to determine whether there is any cause and effect relationship between the effectiveness of DNA lesion repair induced by ionizing radiation and the prognosis for patient survival. If such a correlation is found, it could become one of the parameters by which we decide whether to repeat radiotherapy or give it up in cases which seem doubtful from a clinical point of view. If the levels of 8-oxoGua and 8-oxoGdG excreted in urine are the result of oxidative DNA damage repair, is there any correlation between the clinical effects of radiotherapy and the results of quantitative assays of these markers? In our study (53) we analysed urinary excretion of a wide range of parameters: 8-oxoGdG, 8-oxoGua as well as 8-oxoGdG levels in blood leukocyte DNA in patients diagnosed with head and neck cancer in the course of radiotherapy. In a small subpopulation of 10 patients (about 37%), a significant increase was noted in 8-oxoGdG in cellular DNA along with an increase in 8-oxoGua in the excreted urine on completion of radiotherapy. Since 8-oxoGua is the product of DNA damage repair, it is possible that for some patients undergoing treatment a combination of low hOGG1 (7,8-dihydro-8-oxoguanine glycosylase) (the main DNA damage repair enzyme in humans) activity and radiotherapy is associated with an increased level of 8-oxoGua in intracellular DNA. The detailed clinical analysis of the subgroup of cases involving patients with increased levels of modified guanine in their urine showed significantly better prognoses than other cases.

Based on this observation in the ground-breaking study (54), we tried to identify the prognostic marker of radiotherapy from among the different parameters that describe oxidative DNA damage. A comparison of the levels of the derivatives with the parameters that indicate therapeutic success, such as overall survival, allowed us to infer a causal relationship between the efficacy of repair of ionizing radiation-induced DNA damage and the prognosis for the patient.

Examinations of the test parameters revealed a significant increase in the survival rate of patients with increased urinary excretion of 8-oxoGua accompanied by unaltered 8-oxoGdG levels in leukocyte DNA in samples collected 24 hours after the first fraction of radiation therapy as compared to baseline values (overall post-treatment survival of 60 months in 50% of patients meeting these criteria vs. 10% of patients not meeting these criteria).

In conclusion, it should be stated that increased urinary excretion of 8-oxoGua following the first fraction of radiation therapy accompanied by unaltered levels of 8-oxoGdG in the DNA of peripheral blood leukocytes may be a parameter that defines the success of radiation therapy (Figure 3).
8-oxoGua- cancer biomarker and prognostic factor

Figure 4. Daily urine excretion of 8-oxoGua (p=0.004), 8-oxodG (p=0.001) in the control group of healthy subjects and in all patients before treatment (median values) with levels of 8-oxodG in the DNA (p=0.002) isolated from venous blood leukocytes in the control group of healthy subjects and in the group of cancer patients prior to treatment (21).

6.3. Oxidative DNA damage repair products as molecular markers of cancer

The results of studies by several authors, comparing the amounts of oxidative stress markers excreted with urine in patients diagnosed with neoplasms and in healthy individuals, indicate higher levels of these modifications in cancer patients. This significant increase in the quantity of the analysed markers of oxidative DNA lesions may reflect the conditions of oxygen shock associated with neoplastic diseases (55,56). Cancer cells are characterised by consistently elevated levels of ROS, and they can therefore be particularly sensitive to oxidative stress intensity. ROS may therefore result in cell apoptosis (13). It is also possible that elevated levels of ROS are connected with oncogenesis mediated by oxidative DNA lesions. Neoplastic disease can elevate the level of oxidative stress in the body and therefore contribute to 8-oxoguanine production. An increased concentration of DNA oxidation products was found to be associated with a greater advancement of lung cancer, and both radiotherapy and chemotherapy increased the parameters of oxidative DNA damage (57). Moreover, in patients with lung cancer, a correlation was found between the intensity of the oxidative stress and the progression of the disease (58). Another study found that 8-oxodG urine content decreased in patients with small-cell lung cancer (SCLC) who were partly and totally responsive to chemotherapy and increased in patients in whom the disease had progressed (36).

We conducted (21) a study to compare the levels of the assayed biomarkers in cancer patients (n=222) and healthy volunteers (n=134). The study included a group of patients with different types of cancer, namely, head and neck cancer (n=45), breast cancer (n=32), colon cancer (n=25), lung cancer (n=37), endometrial cancer (n=15), ovarian cancer (n=39), testicle cancer (n=7), prostate cancer (n=11), and gastrointestinal cancer (n=11).

The results suggest that oxidative stress in patients with malignant neoplasms, as evidenced by increased urine levels of the assayed modifications, may be characteristic not only of the affected tissue, but of other tissues as well as the entire body (Figure 4).

An assay allowing us to determine the levels of the basic oxidative stress markers could potentially be used in clinical practice as an additional, auxiliary marker in cancer diagnostics.

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**Key Words:** oxidative DNA damage, biomarkers, 8-oxoGua, 8-oxodG, prognostic factors, cancer patients.

**Send correspondence to:** Krzysztof Roszkowski, Department of Radiotherapy, the F. Lukaszyk Oncology Center Bydgoszcz, Romanowskiej 2, 85-796 Bydgoszcz, Poland, Tel: 4852-3743744, Fax: 4853-3743568, E-mail: roszkowskik@co.bydgoszcz.pl